Kenaf Phytoalexin: Toxicity of *o*-Hibiscanone and Its Hydroquinone to the Plant Pathogens *Verticillium dahliae* and *Fusarium oxysporum* f. sp. *vasinfectum*

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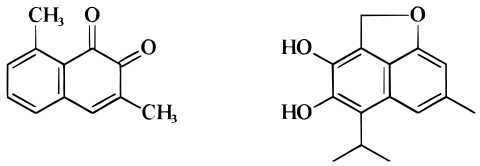
o-Hibiscanone (HBQ) is a phytoalexin produced by kenaf in response to infection by the wilt pathogen *Verticillium dahliae*. In several bioassays utilizing both conidia and mycelia of *V. dahliae*, HBQ was significantly more toxic than desoxyhemigossypol, the most potent phytoalexin produced by cotton. HBQ also was more toxic to conidia of *Fusarium oxysporum* f. sp. *vasinfectum*. ^{13}C-NMR experiments revealed that HBQ is reduced to its hydroquinone by *V. dahliae* conidia. Bioassays of HBQ and its hydroquinone established that the quinone is considerably more toxic to *V. dahliae* than is the hydroquinone. This biotransformation apparently represents a detoxification mechanism employed by the pathogen.

Keywords: *Hibiscus cannabinus; fungicide; cotton; Gossypium; phytoalexin detoxification*

INTRODUCTION

Verticillium wilt caused losses to the U.S. cotton industry in 1996 that were estimated to be in excess of $95 million (Blasingame, 1996). In addition to its effect on yield, the disease also reduces cotton fiber quality (with respect to length, uniformity, and strength) and seed weight and vigor (Bell, 1992). Seabrook Sea Island (SBSI) 12B-2 (*Gossypium barbadense*) has been considered to be the cotton variety with the highest level of resistance to *Verticillium dahliae*. This high level of resistance is attributed to the speed of phytoalexin (PA) synthesis relative to the speed of secondary colonization by the fungus and to the quality of the PAs produced. Unpublished studies in our laboratory have shown that the new Upland cotton varieties selected for resistance to *V. dahliae*, such as Acala Prema (*G. hirsutum*), have response times that are nearly the same as those observed in SBSI. To achieve a significant increase in resistance to *V. dahliae*, it appears likely that PAs from outside the *Gossypium* genus may be required.

Idessis (1966) measured the resistance of 15 plant species to 20 strains of *Verticillium* isolated from 17 plant species. Kenaf, camomile, alfalfa, and snapdragon were found to be highly resistant to most isolates tested. Kenaf (*Hibiscus cannabinus*), being in the same family as cotton (i.e., Malvaceae), is of particular interest to us. Four varieties of kenaf had fresh leaf weights 2 weeks after inoculation with a defoliating strain of *V. dahliae* that were 89% of those of the uninoculated controls (Stipanovic et al., 1998), whereas leaf weights of infected Acala Prema cotton was reduced to 59% of the controls. An investigation of kenaf led to the isolation and identification of a new PA, *o*-hibiscanone (HBQ) (Figure 1) (Bell et al., 1998).



o-Hibiscanone Desoxyhemigossypol

Figure 1. Structures of kenaf phytoalexin *o*-hibiscanone and cotton phytoalexin desoxyhemigossypol.

In this paper, the ED_{50} for HBQ to both *V. dahliae* and *Fusarium* f. sp. *oxysporum vasinfectum* (*F.o.v.*) conidia, as determined by turbimetric bioassays in buffered nutrient media, is reported and compared to that of the most potent cotton PA desoxyhemigossypol (dHG). Additionally, the toxicities (LD_{100} values) of HBQ to *V. dahliae* conidia under both aerobic and anaerobic conditions are reported, and the toxicities of HBQ and dHG (Figure 1) are compared against mycelia of four isolates of *V. dahliae* (two isolates from the defoliating pathotype and two isolates from the nondefoliating pathotype on cotton). The biotransformation of HBQ to its dihydroquinone by *V. dahliae* conidia was demonstrated. Last, the toxicity of HBQ versus that of its dihydroquinone was evaluated under anaerobic conditions.

MATERIALS AND METHODS

Fungal Culture. Conidial suspensions of *V. dahliae* Kleb. (isolate V-76, a defoliating pathotype to cotton) were prepared on the day of the experiment as follows. Suspensions of conidia in deionized water were harvested from 3-day-old cultures started by spreading conidia on PDA at room temperature (~22 °C). The suspensions were filtered through Whatman No. 4 filter paper to remove mycelial fragments, and the suspended conidia were washed three times by centrifuging from 50 mL of sterile deionized water. For the bioassay

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Table 1. Toxicity of HBQ to Conidia of *V. dahliae* and *F.o.v.* As Determined by Turbidity Measurements[a]

V. dahliae (V-76)		*F.o.v.* (isolate 5)	
μg of HBQ/mL	% growth inhibition[b]	μg of HBQ/mL	% growth inhibition
		0.6	27 ± 3
0.25	19 ± 5	1.2	43 ± 3
0.80	53 ± 8	1.8	54 ± 3
1.10	71 ± 7	2.4	70 ± 2
2.00	96 ± 2	3.0	84 ± 1

[a] Results are representative of those obtained in three different experiments that were conducted at pH 6.3 in nutrient media containing 1.8% DMSO for 48 h with 10^6 conidia/mL for *V. dahliae* isolate V-76 and *F.o.v.* isolate 5. [b] % growth inhibition = 100 − [(absorbance of treatment/absorbance of control) × 100]. The mean ± standard deviation is given for three conidial suspensions tested separately in one experiment.

Table 2. Toxicity of dHG to Conidia of *V. dahliae* and *F.o.v.* As Determined by Turbidity Measurements[a]

V. dahliae (V-76)		*F.o.v.* (isolate 5)	
μg of dHG/mL	% growth inhibition[b]	μg of dHG/mL	% growth inhibition
		3	14 ± 3
2	12 ± 2	6	24 ± 4
5	28 ± 5	9	31 ± 5
10	56 ± 6	12	49 ± 8
15	94 ± 7	15	76 ± 3

[a] Results are representative of those obtained in three different experiments that were conducted at pH 6.3 in nutrient media containing 0.9% DMSO for 48 h with 10^6 conidia/mL of *V. dahliae* isolate V-76 and *F.o.v.* isolate 5. [b] % Growth inhibition = 100 − [(absorbance of treatment/absorbance of control) × 100]. The mean ± standard deviation is given for three conidial suspensions tested separately in one experiment.

experiments, the suspensions were diluted to a final concentration of ~10^7 conidia/mL (an absorbance of 0.50 ± 0.05 at 600 nm). For the HBQ reduction experiments, the suspensions were first diluted to 10^7 conidia/mL and then concentrated via volume reduction to 6.5×10^8 conidia/mL. The *V. dahliae* and *F.o.v.* cultures used in these studies are maintained in the *Verticillium* and *Fusarium* collections of the Cotton Pathology Research Unit, Southern Crops Research Laboratory, USDA, College Station, TX.

Turbimetric Bioassay. Turbimetric bioassays for restriction of growth by PAs were performed as previously described (Zhang et al., 1993) in a defined nutrient medium containing 30 mM $NH_4H_2PO_4$, 115 mM D-glucose, 1.0 mM $MgSO_4 \cdot 7H_2O$, 75 mM KH_2PO_4, and 75 mM Na_2HPO_4 (pH 6.3). HBQ or dHG was dissolved in dimethyl sulfoxide (DMSO) and then diluted with nutrient medium to yield the appropriate PA concentration in a 2% solution of DMSO for HBQ or a 1% solution of DMSO for dHG. Aliquots of this solution were then diluted with nutrient medium containing 2% DMSO (or 1% DMSO for dHG) to yield a series of solutions with decreasing PA concentrations. *V. dahliae* (isolate V-76) or *F.o.v.* (isolate 5) conidial suspensions (10^7 conidia/mL) were added to each PA solution to yield test solutions with 10^6 conidia/mL and 1.8% DMSO with the appropriate amount of HBQ or 0.9% DMSO with the appropriate amount of dHG. Blank or control test solutions were prepared by adding water or a conidial (*V. dahliae* or *F.o.v.*) suspension, respectively, to aliquots of medium plus DMSO, yielding concentrations identical to those in the PA test solution.

The blank, control, and PA test solutions were each pipetted into 6 wells in a 96-well flat-bottom cluster plate. The plate was incubated in a moist chamber at 25 °C for 48 h, after which time the optical densities (OD) at 550 nm were read on a multiwell plate reader. The mean OD for the six wells containing the blank test solution was subtracted from the mean OD values for the control and PA test solutions. This provided background-corrected values. Relative growth in the treatments as a fraction of the control value was determined by dividing the mean corrected OD of the PA-treated wells by the mean corrected OD of the control. This value was determined at each PA concentration. The data were analyzed using a multiple drug−effect analysis program (Chou and Talalay, 1984) to provide the effective dose that reduces growth by 50% (ED_{50} value).

In each experiment, three different conidial suspensions were tested using the same PA solutions, thus allowing an average ED_{50} to be calculated from the three repetitions. The experiments were repeated at least three times. All data shown in Tables 1 and 2 are representative of the experiments as determined using SAS one-way ANOVA (SAS Institute Inc., Cary, NC).

Bioassay of Fungicidal Activity against *V. dahliae* Conidia in Air. Bioassays to determine the fungicidal activity of HBQ and dHG against *V. dahliae* conidia in water during a 1 h exposure were performed as follows. A weighed amount of HBQ or dHG PA (PA) was dissolved in DMSO, and the resulting solution was diluted with water to yield the desired PA concentration in 2% DMSO. Aliquots of this solution were then diluted with water containing 2% DMSO to yield a series of solutions with decreasing PA concentration but the same level of DMSO. Conidial suspension (10^7 conidia/mL) was added to each PA solution to give test solutions with 10^6 conidia/mL and 1.8% DMSO plus 4, 8, 12, 16, 20, or 24 μg/mL PA. A blank and a control were prepared by adding water or a V-76 suspension, respectively, to aliquots of water with 2% DMSO, yielding solution blank and control conidia concentrations identical to those in the PA test solutions. After 1 h, the PA and blank test solutions were diluted (1:240). The dilution procedure was replicated four times for the control test solution. The dilute test solutions were pipetted onto separate PDA plates and spread to evenly distribute the conidia. The plates were incubated at room temperature for 5 days, after which time the number of fungal colonies on each plate was counted. The blank was used to check for contamination in the water or water plus DMSO solutions used in the bioassay.

From the colony counts and the dilution factor, the concentrations of live conidia in the PA and control test solutions were calculated. A mean concentration of conidia in the control was obtained from the four replicates, and this value was used to calculate the percentage of live conidia in each PA test solution. Four different V-76 conidial suspensions were tested against each PA solution in each experiment. The experiment was repeated three times, and the data sets were analyzed for statistical significance using SAS one-way ANOVA (SAS Institute).

Bioassay of Fungicidal Activity against *V. dahliae* Conidia under Nitrogen. The toxicity of HBQ to *V. dahliae* conidia in water over 1 h under a nitrogen atmosphere was determined in a manner similar to the aerobic bioassay, with the following modifications: after preparation, the HBQ, control, and blank solutions along with the conidial suspension and the water used in the dilutions were thoroughly deaerated with nitrogen, tightly sealed, and placed into a glovebox that was backfilled and flushed with nitrogen. With a continuous flow of nitrogen into the box and at 1 min intervals, a conidial suspension was added to the HBQ solutions and control and water was added to the blank. After 1 h, these test solutions were diluted 1:240, and the dilute solutions were removed from the glovebox. The rest of the bioassay and data analysis were carried out as described for the aerobic procedure. Four different conidial suspensions were tested against each HBQ solution in each experiment, and the experiment was repeated three times. Statistical analysis of the results was carried out via SAS one-way ANOVA (SAS Institute).

Bioassay of Fungicidal Activity against *V. dahliae* Mycelia. Bioassays were carried out as previously described (Mace et al., 1990). Two isolates (PH and TS-2) that are nondefoliating pathotypes and two isolates (V-76 and V-44) that are defoliating pathotypes on cotton were tested.

Bioassay of HBQ versus Its Dihydroquinone. The relative toxicities of HBQ and its hydroquinone (HBQ-Hq) were

determined in a bioassay performed over 2 days. On the first day, concentrated solutions of HBQ and reduced glutathione (GLUT) were prepared by dissolving the solid compounds in DMSO and then diluting with water to give 2% DMSO. Aliquots of the HBQ concentrate, GLUT concentrate, and water containing 2% DMSO were thoroughly deaerated with nitrogen, tightly sealed, and placed in a glovebox backfilled with nitrogen. With a continuous flow of nitrogen into the box, GLUT concentrate was added to an aliquot of HBQ concentrate and left to sit overnight to yield the dihydroquinone (HBQ-Hq + GLUT) solution.

On the second day, a conidial suspension of *V. dahliae*, isolate V-76, was prepared, deaerated with nitrogen, sealed, and placed into the glovebox. Water used in the dilution procedure also was deaerated and put into the box. With a continual flow of nitrogen into the box, aliquots of GLUT concentrate and HBQ concentrate were mixed with water plus DMSO to give individual GLUT and HBQ solutions with the same concentrations of chemicals as in the DHQ + GLUT solution made the first day. Two portions of the water plus DMSO were reserved for the blank and control. To start the bioassay test sequence, an aliquot of GLUT concentrate and one of the conidial suspension (10^7 conidia/mL) were added to a portion of HBQ concentrate to give a HBQ + GLUT test solution. A sample of this test solution was immediately removed and diluted. The addition of conidial suspension followed by sampling and dilution was then carried out on the control, GLUT, HBQ, and HBQ-Hq + GLUT solutions. In the case of the blank, water was added instead of the conidial suspension. Conidia were exposed to the PAs for 15 and 30 min and 1 h. When applicable, the concentrations in the test solutions were 1.8% DMSO, 40 μg/mL HBQ or HBQ-Hq, 132 μg/mL GLUT, and 10^6 conidia/mL.

The dilute test solutions were pipetted onto a PDA plate and then smeared to evenly distribute the conidia. The plates were incubated at room temperature for 5 days, after which time the number of fungal colonies on each plate was counted. The blank was used to check for contamination in the water or water plus DMSO used in preparing the concentrates or other solutions. From the dilution factor and the colony counts on the plates, the concentrations of live conidia in the control, GLUT, HBQ, HBQ-Hq + GLUT, and HBQ + GLUT test solutions at each sampling time were calculated. The control conidial concentrations obtained from the four sampling times were used to calculate a mean control conidial concentration. This mean concentration and the live conidial concentrations in the GLUT, HBQ, HBQ-Hq + GLUT, and HBQ + GLUT test solutions were used to calculate the percentage of live conidia in each test solution. Any conidial concentration within the standard deviation of the mean control concentration was considered to indicate 100% live conidia. The experiment was repeated three times, and the results were combined.

Biological Reduction of HBQ: NMR Experiment. Standard ^{13}C-NMR spectra for HBQ and its hydroquinone were obtained on a Bruker ARX-300 instrument. The HBQ spectrum was obtained from a solution containing 0.75 μg/mL HBQ in 44% DMSO-d_6 and 56% D_2O, and the hydroquinone spectrum was obtained from a similar HBQ solution that had been reduced with $Na_2S_2O_4$ (i.e., the concentrations were 0.75 μg/mL HBQ and 2.67 μg/mL $Na_2S_2O_4$ in 44% DMSO-d_6 and 56% D_2O).

The reduction of HBQ by conidia of *V. dahliae*, isolate V-76, also was investigated by ^{13}C-NMR experiments. Solutions of ^{13}C-labeled HBQ (0.1 mg/mL) in DMSO and D_2O were prepared and placed into 10 mm NMR tubes. Noting the time, aliquots of *V. dahliae* conidial suspension were added to the tubes under nitrogen to give a final concentration of 6.5×10^7 cells/mL. Immediately after addition of conidia, one tube of solution was heated in boiling water for 3 min to kill the conidia, cooled to room temperature, and then analyzed by ^{13}C NMR. After a 15 min interval, a second tube of solution was boiled, cooled, and analyzed. This procedure was repeated at 35, 70, and 140 min time points. Changes in HBQ and/or its dihydroquinone were assessed via observation of the appropriate ^{13}C peaks as determined from the standard ^{13}C-NMR spectra obtained previously.

The effects over time of reduced glutathione on HBQ were evaluated via visual observation of solution color and changes in the ^{13}C-NMR spectrum of HBQ. Aliquots of GLUT solutions were added under nitrogen to 10 mm NMR tubes containing ^{13}C-HBQ giving 0.18 μg/mL HBQ in 10−14% DMSO-d_6 and water. This gave a ratio of 1:1.5 equivalents of HBQ to reductant. The tubes were sealed with a backfill of nitrogen, and then color assessment and ^{13}C-NMR analysis were carried out every 20 min until no further changes in either the color or the spectrum were observed. As a check, the viability of *V. dahliae* conidia treated as above for 140 min with D_2O and DMSO was evaluated and found to be no different from a similar treatment in H_2O and DMSO.

Table 3. Percentage of Conidia of *V. dahliae* Killed by dHG and HBQ As Determined by Colony Counts on PDA Plates[a]

concn[b] (μg/mL)	aerobic		anaerobic[d]
	dHG	HBQ	HBQ
4		12 ± 10[c] (a)	10 ± 7 (a)
8	0 ± 0 (a)	43 ± 12 (b)	44 ± 12 (b)
12	0 ± 0 (a)	90 ± 8 (c)	96 ± 4 (c)
16	24 ± 6 (b)	99 ± 1 (c)	99 ± 1 (c)
20	45 ± 5 (c)	100 ± 1 (c)	100 ± 1 (c)
24	68 ± 2 (d)	100 ± 0 (c)	100 ± 0 (c)

[a] Results are representative of those obtained in three different experiments that were conducted in water for 1 h on *V. dahliae* isolate V-76. [b] Compound solutions contained 1.8% DMSO. [c] Mean percentage of conidia killed and standard deviation calculated from four cell suspensions tested against the same compound solution in one experiment. The same letter in parentheses within a column indicates that kill levels are not statistically different as established by ANOVA analysis. [d] Conidia were exposed to HBQ under a nitrogen atmosphere, diluted, and then aerobically spread on PDA plates.

RESULTS

Aerobic Bioassays. We previously used a turbimetric method to evaluate the growth-inhibiting properties of dHG and other cotton PAs to *F.o.v.* (Zhang et al., 1993). Because dHG was the most toxic cotton PA, its toxicity was compared to that of HBQ from kenaf (Tables 1 and 2). From these results, ED_{50} values (Chou and Talalay, 1984) were calculated. For dHG they were 5.8 ± 0.8 and 11.5 ± 1.4 μg/mL against *V. dahliae* and *F.o.v.*, respectively. For HBQ they were 0.47 ± 0.04 and 1.37 ± 0.11 μg/mL against *V. dahliae* and *F.o.v.*, respectively, yet this bioassay does not differentiate between fungicidal and fungistatic activity.

An alternative bioassay was utilized to measure fungicidal activity. In an initial study (Stipanovic et al., 1998), we found that exposure of conidia to HBQ for periods of 15 min to 1 h provided a maximum fungicidal rate, with 99% of the conidia killed after 15 min. On the basis of this preliminary experiment, we exposed the conidia to various concentrations of HBQ and dHG for 1 h. Conidia were then diluted and spread on PDA plates. Colonies were counted as a measure of surviving conidia after 5 days. The results of these tests are shown in Table 3. The ED_{50} values for dHG and HBQ in this bioassay were calculated to be 20.7 and 7.0 μg/mL, respectively.

We previously reported the fungicidal activity of dHG to mycelia of four isolates of *V. dahliae* (Mace et al., 1990). Two of these isolates (i.e., PH and TS-2) were from the nondefoliating pathotype on cotton, and two isolates (i.e., V-44 and V-76) were from the defoliating pathotype. We have repeated this experiment to com-

Table 4. Fungicidal Activity of dHG and HBQ to Mycelia of Two Nondefoliating Isolates and Two Defoliating Isolates of *V. dahliae* [Adapted from Stipanovic et al. (1998)]

concn[d] (μg/mL)	percentage of culture wells with live mycelia[a]							
	PH[b]		TS-2[b]		V-44[c]		V-76[c]	
	dHG	HBQ	dHG	HBQ	dHG	HBQ	dHG	HBQ
0	100	100	100	100	100	100	100	100
5	ND	36	ND	61	ND	25	ND	33
10	100	6	100	3	100	6	100	6
15	64	3	56	0	22	3	42	0
20	0	ND	0	ND	0	ND	0	ND

[a] Mean percentage; three experiments each with 12 samples per concentration per PA; ND = not determined. [b] PH and TS-2 are cotton nondefoliating isolates of *V. dahliae*. [c] V-44 and V-76 are cotton defoliating isolates of *V. dahliae*. [d] pH 6.3 nutrient solutions with 1% DMSO for dHG and 2% DMSO for HBQ.

pare HBQ with dHG, and the results are shown in Table 4. HBQ was almost twice as toxic as dHG to mycelia of the four *V. dahliae* isolates. dHG required 20 μg/mL to kill all mycelia, whereas HBQ required only 10 μg/mL to kill almost all of the mycelia. The nondefoliating and defoliating isolates did not show any differences in sensitivity to either PA.

Biotransformation of HBQ. When [11-^{13}C]-HBQ was mixed with *V. dahliae* conidia, the solution color changed over a period of ~2 h from intense yellow-orange to colorless. During this time, the C-11 ^{13}C-NMR peak at 23.64 ppm slowly disappeared and a peak at 25.06 ppm grew to a maximum. The yellow color could be restored by aerating the colorless solution. The biotransformed product was shown to be the hydroquinone of HBQ by comparing its ^{13}C-NMR spectrum with that obtained by reducing HBQ with sodium dithionite.

Anaerobic Bioassays. There was no effect on the survival of *V. dahliae* conidia when they were treated with the HBQ hydroquinone (40 μg/mL) for 1 h, but the same concentration of the parent HBQ killed all conidia in 15 min. ANOVA statistical analysis established that the toxicity for HBQ under anaerobic conditions was not different from that observed under aerobic conditions (Table 3).

DISCUSSION

The ED_{50} values for dHG against *F.o.v.* (11.5 μg/mL) and *V. dahliae* (5.82 μg/mL) were 8−12 times greater than those of HBQ (0.47 and 1.37 μg/mL, respectively), indicating that HBQ is a much more effective inhibitor of growth than is dHG.

In the bioassay for fungicidal activity in which *V. dahliae* conidia were exposed to the PA for 1 h, the kenaf PA HBQ killed 99% of the conidia at 16 μg/mL, whereas the cotton PA dHG killed only ~25% of the conidia at this concentration. In a mycelia bioassay against the two nondefoliating pathotypes PH and TS-2 and against the two defoliating pathotypes V-76 and V-44 of *V. dahliae*, HBQ was significantly more toxic, killing all of the mycelia in ~66% of the culture wells at 5 μg/mL, whereas dHG killed all of the mycelia in 33% of the wells at 15 μg/mL. No significant differences in sensitivity to HBQ were observed between the defoliating and nondefoliating pathotypes.

A ^{13}C-NMR study showed that HBQ was reduced to the hydroquinone by *V. dahliae*, and bioassays revealed that the hydroquinone of HBQ is nontoxic to *V. dahliae* conidia at the highest concentration tested (40 μg/mL). Therefore, it can be concluded that the reduction of HBQ to the hydroquinone represents a detoxification of this PA by *V. dahliae*. Medentser and Akimenko (1998) have proposed that naphthoquinones effect their cytotoxicity by interfering with the normal respiratory chain by the uncontrolled oxidation of NADH and NADPH, by the formation of reactive oxygen species, and by formation of free radicals that interact with DNA and RNA. These mechanisms would account for the low toxicity of the hydroquinone of HBQ under anaerobic conditions. In the 1 h exposure bioassay, the toxicity of HBQ was undiminished under anaerobic conditions (Table 3).

These results show that HBQ is a potent PA that is significantly more toxic to both *V. dahliae* and *F.o.v.* than the most effective PA produced by cotton. These results support the finding that kenaf is more resistant to verticillium wilt than is cotton (Stipanovic et al., 1998). Thus, PAs in cotton and kenaf appear to represent an essential defense mechanism in resistance to *V. dahliae*. Furthermore, increasing PA potency appears to offer an effective avenue to enhancing resistance to wilt fungi.

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